



THE UNIVERSITY *of* EDINBURGH

Edinburgh Research Explorer

Potential of genomic technologies to improve disease resistance in molluscan aquaculture

Citation for published version:

Potts, R, Gutierrez Silva, A, Penaloza, C, Regan, T, Bean, T & Houston, R 2021, 'Potential of genomic technologies to improve disease resistance in molluscan aquaculture', *Philosophical Transactions of the Royal Society B: Biological Sciences*, vol. 376, no. 1825. <https://doi.org/10.1098/rstb.2020.0168>

Digital Object Identifier (DOI):

[10.1098/rstb.2020.0168](https://doi.org/10.1098/rstb.2020.0168)

Link:

[Link to publication record in Edinburgh Research Explorer](#)

Document Version:

Peer reviewed version

Published In:

Philosophical Transactions of the Royal Society B: Biological Sciences

General rights

Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy

The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact openaccess@ed.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.



Potential of genomic technologies to improve disease resistance in molluscan aquaculture

Robert W.A. Potts^{1,2}, Alejandro P. Gutierrez¹, Carolina S. Penaloza¹, Tim Regan¹, Tim P. Bean¹, Ross D. Houston¹

1. The Roslin Institute and Royal (Dick) School of Veterinary Studies, University of Edinburgh, Midlothian EH25 9RG, United Kingdom
2. Centre for Environment, Fisheries and Aquaculture Science (Cefas), Weymouth Laboratory, Dorset DT4 8UB, United Kingdom

Abstract

Molluscan aquaculture is a major contributor to global seafood production, but is hampered by infectious disease outbreaks which can cause serious economic losses. Selective breeding has been widely used to improve disease resistance in major agricultural and aquaculture species, and has clear potential in molluscs, albeit its commercial application remains at a formative stage. Advances in genomic technologies, especially development of cost-efficient genomic selection, have potential to accelerate genetic improvement. However, tailored approaches are required due to the distinctive reproductive and lifecycle characteristics of molluscan species. Transgenesis and genome editing, in particular CRISPR/Cas systems, have been successfully trialled in molluscs, and may further understanding and improvement of genetic resistance to disease through targeted changes to the host genome. Whole organism genome editing is achievable on a much greater scale compared to other farmed species, making genome-wide CRISPR screening approaches plausible. This review discusses the current state and future potential of selective breeding, genomic tools, and genome editing approaches to understand and improve host resistance to infectious disease in molluscs.

Key words: Mollusc, selective breeding, gene editing, disease resistance, transgenesis, CRISPR/Cas9

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Introduction

Aquaculture plays a major role in global food security, producing 80.1 million tonnes of food in 2016. Growth in the aquaculture sector is continuing and has become an increasingly important source of seafood as capture fisheries have remained relatively stable since 1990 [1, 2]. Molluscs, including bivalves, gastropods and cephalopods represent 21.4 % of all aquaculture globally, but avenues for further development remain due to their fundamentally different biological features compared to finfish aquaculture species [1]. Their extremely high fecundity, tolerance to diverse environments, and minimal input requirements means molluscan aquaculture has major potential for efficient animal protein production [2, 3]. Bivalves such as oysters, clams, mussels and scallops provide the bulk of molluscan aquaculture, with gastropods such as abalone and freshwater snails also making a significant contribution. Cephalopod aquaculture currently has very little commercial significance, possibly due to lack of culture expertise [4] unknown optimal nutritional requirements, and difficulties associated with animal reproduction in captivity [5]. The distribution of molluscan aquaculture is mostly focused in Asia, especially China where mollusc production accounts for more than 66 % of total aquaculture output by live weight (Figure 1) [6, 7].

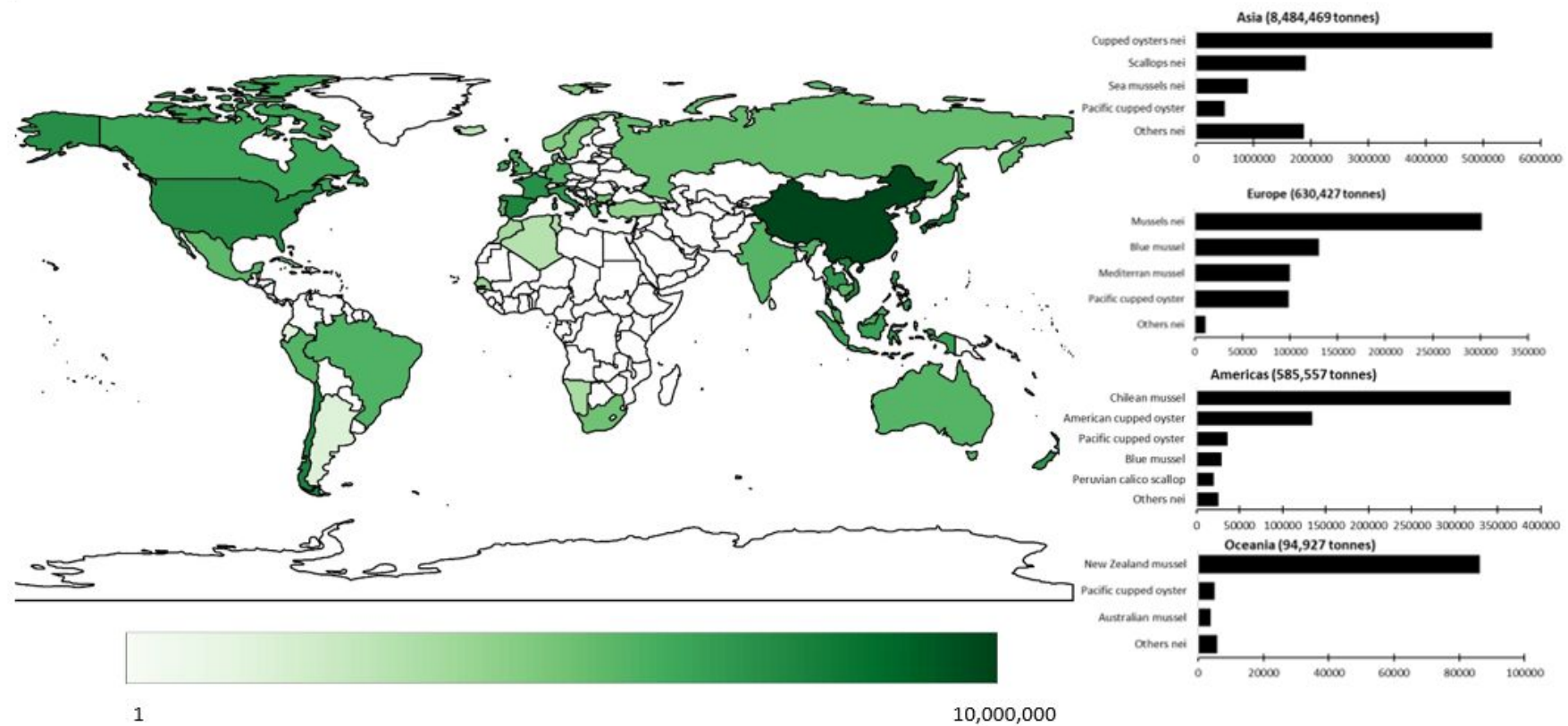


Figure 1) Global mollusc production in 2018 using FAO data (live weight tonnes log₁₀). Bar charts show major production focal species across Americas, Europe, Asia and Oceania according to FAO. nei = not elsewhere included

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

The grow-out phase for the majority of molluscan aquaculture occurs in the open ocean environment, where animals are exposed to a wide range of viruses, bacteria, fungi, protozoans and metazoans that can cause infectious disease outbreaks [8-10]. Such disease issues can seriously constrain the sustainability and expansion of molluscan aquaculture. While assessing the impact of specific pathogens on molluscan aquaculture is challenging, one pertinent example is the devastating impact of the ostreid herpesvirus (OsHV) which is wide spread and impacts most oyster producing countries, particularly the microvariant OsHV-1 μ var [11]. Multi-faceted approaches are required to prevent and mitigate the impact of such outbreaks, especially considering potential increases in disease risk due to climate change [12]. Due to its economic and animal welfare importance, disease resistance is arguably the most important target trait in the breeding goals of advanced aquaculture breeding programmes [13-16]. Furthermore, in many cases alternative means of disease prevention are lacking, and traditional vaccination approaches are impossible in molluscan aquaculture due to open culture systems and the lack of an adaptive immune system [17].

Genetic improvement of disease resistance via selective breeding without negative consequences of reduced genetic diversity requires a well-organised breeding programme, including routine trait recording and tracking of pedigree [18]. There has been significant research effort and a number of such breeding programmes for molluscs, but the majority of molluscan aquaculture is still based on wild-sourced seed [18, 19]. Therefore, as with finfish species, there is significant untapped potential to improve production via well-managed selective breeding for genetic improvement of target traits [1, 20], which has been successfully demonstrated in molluscs [21-25]. An increasing number of genomic tools have been developed, including high quality reference genomes and single nucleotide polymorphism (SNP) arrays. These have led to new approaches to map loci affecting disease resistance, and

1
2
3 studies demonstrate the potential of marker assisted selection and particularly genomic
4 selection to expedite genetic gain for target traits [18]. Genetic engineering and genome editing
5 approaches (such as CRISPR/Cas9) have further potential to improve disease resistance, but
6 have not yet been widely tested or applied in molluscs. Certain aspects of molluscan biology
7 and life history traits present unique challenges and potential for genetic improvement with
8 exciting opportunities to understand and improve disease resistance. This review will focus on
9 the developments and applications of selective breeding and genome editing technologies to
10 date, and discuss future innovations that are likely to have significant impacts on prevention of
11 disease and improvement of molluscan aquaculture production in the future.
12
13
14
15
16
17
18
19
20
21
22
23

24 **Disease resistance traits in molluscs**

25
26 To improve disease resistance via genetic and genomic approaches, it is important to define
27 and understand the definition of the target traits. Disease resistance is an encompassing term
28 that from a biological perspective can describe a spectrum ranging from increased tolerance to
29 complete protection. Both of these outcomes are beneficial to production and can be targets for
30 improvement using selective breeding [26]. For molluscs, disease resistance in the broad sense
31 is typically measured as the survival of animals in response to a challenge, either an
32 experimental challenge via immersion or injection of a pathogen, or a 'field' challenge where
33 survivors of a particular outbreak are sampled and considered as resistant. In some cases,
34 including where the pathogen or parasite does not kill the host, counts of the pathogen per
35 animal provide an appropriate trait to measure, with low pathogen burden considered as more
36 resistant [27, 28].
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52

53 While these measures of disease resistance are likely to be practically effective, as has been
54 shown for finfish aquaculture species [29], understanding the molecular mechanisms
55 underlying the resistance traits is more challenging. This is particularly the case for molluscan
56
57
58
59
60

species, as the mechanisms of invertebrate immunity are not as well understood as for vertebrates [30], especially given the range of host species and pathogens that impact aquaculture production. Molluscs lack adaptive immunity and instead primarily utilise non-specific responses to pathogens [17, 30]. Autophagy, phagocytosis, and other generalised defence mechanisms play a key role in the response to infection in several mollusc species [31]. Furthermore, anti-viral priming can provide an innate immunity upon reinfection, which has been demonstrated in oysters [32, 33]. However, while the cellular mechanisms of immune response and disease resistance require further research, the rapidly expanding genomic toolbox will assist in meeting this challenge (see ‘Towards functional mechanisms underpinning genetic resistance in molluscs’ below).

Selective breeding for disease resistance

Disease resistance traits have been demonstrated in several studies to be heritable, and selective breeding via family selection and mass selection has been successful in improving resistance in several oyster species (Table 1) [23]. The traits have been improved via a mix of experimental challenges and survival data collected from field outbreaks, with reasonably high genetic correlations observed between the traits measured in the two disparate environments [34], which bodes well for the use of experimental challenge models to reflect disease resistance in commercial settings. The focus of the diseases and pathogens for such studies have largely been those that cause high levels of mortalities in commercial farming, such as OsHV-1, *Bonamia spp.*(Bonamiosis), *Haplosporidium nelsoni* (MSX), and *Perkinsus marinus* (Dermo) [35-41], which can have additional negative impacts via restrictions on movement of stocks.

Table 1) Heritability estimates for disease resistance in molluscs since 2015 (see Degremont et al., 2015 [23])

Host species	Pathogen	Type	Organisation / Location	Method	Heritability estimate (range)	References
<i>Crassostrea gigas</i> / Pacific oyster	OsHV	Industrial	MPI and Cawthron - New Zealand	Field survival and laboratory virus challenge survival of hatchery broodstock	0.33 - 0.56	[34]
<i>Crassostrea gigas</i> / Pacific oyster	OsHV	Experimental	Oregon State University - Tomales bay, USA	Field survival and laboratory virus challenge survival of hatchery broodstock imported from France	0.22 - 0.81	[42]
<i>Crassostrea gigas</i> / Pacific oyster	OsHV	Experimental	Cefas and Roslin Institute - UK	Laboratory virus challenge survival of hatchery broodstock	0.08 - 0.25	[25]
<i>Haliotis rufescens</i> / red abalone	Candidatus <i>Xenohaliotis californiensis</i>	Experimental	CEAZA and Universidad Católica del Norte - Chile	Measurement of disease intensity in hatchery reared from commercial stock	0.21 - 0.36	[27]
<i>Crassostrea gigas</i> / Pacific oyster	<i>Vibrio aestuarianus</i>	Experimental	IFREMER - France	Laboratory bacterial challenge of hatchery broodstock	0 - 1.05	[43]
<i>Pinctada fucata</i> / Pearl oyster	<i>Polydora ciliata</i>	Industrial	Chinese Academy of Fishery Sciences	Field challenge of hatchery broodstock	0.21 - 0.47	[44]
<i>Crassostrea gigas</i> / Pacific oyster	OsHV and <i>Vibrio aestuarianus</i>	Experimental	IFREMER - France	Field survival and laboratory challenge of hatchery broodstock at different life stages	0 - 2.58	[45]
<i>Crassostrea gigas</i> / Pacific oyster	OsHV	Experimental	IFREMER - France	Field survival and laboratory virus challenge survival of hatchery broodstock	0.49 - 0.75	[24]

The significant heritability estimates for disease resistance traits highlights the potential to improve resistance in farmed mollusc stocks via breeding programmes. Indeed, a survey of mollusc breeding programmes by Hollenbeck and Johnston (2018) highlighted that various family and mass selection approaches are used across the globe, and strikingly high responses to selection have been observed for disease resistance in oysters (~16 % per generation) [18]. These results are comparable with or higher than the typical selection

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

responses observed for performance traits in finfish breeding, and substantially higher than for terrestrial livestock breeding [46]. There are two main potential reasons for this; firstly the high fecundity of mollusc species can facilitate a high selection intensity, and secondly the proximity of mollusc species to wild counterparts which implies significant standing genetic variation for traits important in the farmed environment. The success of both experimental and early-stage commercial breeding programmes for bivalves highlights the potential to make rapid and cumulative genetic gains for target traits [18, 22, 23].

Breeding programme design

Breeding programmes in molluscs must account for unique aspects of their biology and life history, which are strikingly different to livestock and finfish. The extreme fecundity of individual molluscs (up to millions of offspring per cross) provides single cross population sizes that are well in excess of what can be achieved in finfish or agricultural breeding programmes, providing potential for high selection intensity and genetic gain. Mass selection is therefore potentially a viable strategy for improving disease resistance, demonstrated by breeding from survivors of OsHV challenges that resulted in 62% higher survival after four generations of Pacific oysters [47]. Encouragingly, this improved survival was observed both in experimental challenges and in field environments, which highlights the relevance of the experimental challenge model [47]. However, mass selection is unlikely to be a sustainable approach for commercial mollusc breeding. Firstly, it is well established that mass selection can result in inbreeding, particularly in highly fecund species, and this has already been shown in oyster, abalone and scallop species [18]. Secondly, mass selection drastically limits the scope of a breeding programme to primarily focus on one specific trait of interest, losing potentially valuable genetic variation for other traits. Finally, for disease resistance in particular, challenging the candidate broodstock animals with pathogens may be a biosecurity risk, as

hatcheries should be disease free if possible, and survivors may carry and transmit the pathogen.

For most aquaculture species, it is generally considered that the cumulative genetic gains and control of inbreeding offered by family selection is the optimal approach to selective breeding [1]. For disease resistance traits in particular, tracking of individuals and families allows for experimental or 'field' challenges on siblings of the selection candidates, which enables the calculation of breeding values for those candidates without exposing them to the pathogen(s) [48, 49]. This approach is also known as sib-testing (short for sibling-testing) and harnesses the high fecundity of aquaculture species to perform disease challenges of full siblings of candidates. However, tracking individual molluscs can be challenging due to their small size and low value, particularly in the larval stages where survival rates also tend to be low. One approach is to keep families separate during early life stages, although this can confound early-life common environment with genetic effects. Genotyping approaches can be applied for parentage assignment in mixed family environments, albeit the cost of the assay needs to be low and the throughput high to be cost-effective.

While family-based selection offers the cumulative and sustainable genetic gain for production traits of interest, the use of cross-breeding to capitalise on heterosis may also be valuable. Heterosis, or hybrid vigour, has been demonstrated in *Crassostrea gigas* [50], and hybrids of different *Crassostrea* species have been experimentally demonstrated to have beneficial production values compared to single species [51]. One possible reason for the success of cross-breeding in bivalves is that broodstock lines can be relatively inbred, which may result in gains higher than would be otherwise expected [18]. Although the success of experimental heterosis suggests that underlying genetic variation in oysters harbours important traits for culture, the uptake in commercial settings has been limited [52].

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Furthermore, there is currently limited evidence for the application of heterosis for traits related to disease resistance in molluscs, albeit higher general survival has been shown in some cases [53-55]. While substantial knowledge gaps remain, there may be potential to harness the beneficial effects of heterosis within the context of family-based breeding programmes, for example via the breeding and maintenance of specialist lines for cross-breeding, as commonly occurs in the pig breeding sector [56].

Practical applications of breeding for disease resistance

The majority of studies into the genetic basis of disease resistance in molluscs have been focused on the rock oysters *Crassostrea*, which is arguably the most important molluscan aquaculture genus globally (Table 1). Selective breeding for resistance to OsHV-1 in *Crassostrea gigas* conducted by IFREMER in France resulted in consistent improvement across 4 generations in multiple lines, with final survival gains ranging from 43.9 % to 80.6 % compared to control stocks [47]. Progeny derived from these selective breeding programmes have subsequently been used for commercial farming in various European countries [23]. Successes in developing resistance to OsHV are now being applied for resistance to *Vibrio* bacteria, which have been implicated in summer mortality [57]. This work has demonstrated that *Vibrio* resistance can be enhanced by genetic improvement, although the heritability was lower (0.09 – 0.33) than has been reported for OsHV resistance (Table 1). Additionally, little correlation between resistance to *Vibrio* and OsHV was detected which suggests that stocks selectively bred for OsHV resistance maintain potential for improvement to bacterial resistance [45]. Host age was a much more significant factor affecting resistance to either pathogen, which has been suggested previously [52, 58]. It may therefore be important to use animals of a specific age for disease challenges depending on the pathogen.

Breeding programmes for Pacific oyster on the west coast of the USA are targeting improvement in OsHV resistance/survival via family selection [42]. The federally funded Molluscan Broodstock program (MBP) has developed OsHV resistance in oysters collected from the wild in 1996. In the latest field challenges, survival varied from <10 % to >75 % depending on the specific MBP strain, demonstrating that significant gains have been made but there is still potential for improvement [59].

In Australia, a breeding programme instigated by government funded research and now overseen by Australian Seafood Industries (ASI) focuses on genetic improvement of Pacific oysters, and particularly resistance to Pacific oyster mortality syndrome (POMS) caused by OsHV-1. In this programme, 40 to 50 families of oysters are produced each year which are then subjected to both between and within-family selection, in order to maximise genetic gains whilst minimising the impact of inbreeding [60]. Finally, in New Zealand, the Cawthron Institute has run a selective breeding programme for Pacific oysters since 1999. Outbreaks of OsHV in 2010 lead to production of 49 full-sib families in 2013, which descended from survivors to OsHV-1 exposure in the natural environment. These families have formed the basis for the ongoing breeding programme to develop disease resistance in very young spat [22, 61]. In this case, breeding from survivors of family-tracked material has been highly effective, and will capture both within and between family genetic variation in resistance. However, this may not be possible in some breeding programmes where avoiding exposure of broodstock to pathogens is desirable for biosecurity reasons, as is common for aquaculture breeding [13, 15, 48].

While OsHV-1 has been the primary disease resistance target of practical selective breeding programmes to date, resistance to other pathogens and parasites has been targeted for other bivalve species. Dermo and MSX (caused by waterborne parasites *Perkinsus marinus* and *Haplosporidium nelsoni*, respectively) are persistent and highly damaging diseases impacting

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Eastern oyster (*Crassostrea virginica*) production in the USA, where there have been long standing programmes to improve resistance [62]. Survivors of natural outbreaks were used to generate lines of locally resistant oysters, but as individuals were likely exposed to multiple pathogens, it is unclear to what degree resistance to each specific pathogen has been achieved. The resulting lines have been experimentally tested across the range of eastern oyster production and survivorship was generally superior compared to wild oysters, although oysters had greater survival in areas where they were selectively bred making comparison more difficult [63, 64]. Laboratory challenge of oysters with *Perkinsus marinus* revealed that there was a 54.1 % difference in survival between two families in 2014, but no significant family effect was observed when the challenge was repeated in 2015 [65]. This demonstrates that whilst the breeding programmes based on mass selection can achieve significant results, it is more difficult to develop understanding of disease resistance as environmental factors are highly variable.

Beyond *Crassostrea*, promising results from other molluscan aquaculture species include study of genetic variation in resistance and predicted selection response for withering syndrome disease (*Candidatus Xenohaliotis californiensis*) resistance in abalone *Haliotis rufescens* [27], *Listonella anguillarum* resistance in Zhikong scallop *Chlamys farreri* [66], resistance to multiple *Vibrio* species in clam *Meretrix meretrix* [67], and identification of resistance biomarkers for winter mortality/QX protozoan disease caused by *Martelia sydneyi* in *Saccostrea glomerata* [68, 69]. To date, disease resistance is not a major target for mussel breeding, when compared with other molluscs, with no published examples of genetic improvement programmes targeting disease resistance.

While the aforementioned examples of family based breeding programmes have been successful for improving disease resistance in marine molluscs, genomic tools offer many

opportunities for improving breeding programmes, including increased genetic gain. However, this requires a suitable suite of genomic tools to support these activities.

Genomic tools and features

Reference genomes are the basis of modern genomics, as they reveal the structure, organization and functional features of genes, and provide a foundation for molecular and genomic studies [70]. High-quality reference genomes are a useful resource to advance genomic techniques for the genetic improvement of a species, as they facilitate design of genotyping platforms, discovery of molecular genetic variants, and understanding of the genetic architecture of production traits via genome-wide association studies (GWAS). Furthermore, accurate and complete genomes are essential for accurate gene editing techniques (e.g. CRISPR/Cas9) to precisely target genomic loci and prevent off-target editing.

Among commercial bivalve species, draft genomes are currently available for the Pacific oyster *Crassostrea gigas* [71], the Pearl oyster *Pinctada fucata* [72, 73] [74], the Yesso scallop *Patinopecten yessoensis* [75], the Philippine horse mussel *Modiolus philippinarum* [76], the Mediterranean mussel *Mytilus galloprovincialis* [77], the Eastern oyster *Crassostrea virginica* (Genbank Accession Number GCA_002022765.4), the Sydney Rock oyster *Saccostrea glomerata* [78], and the king scallop *Pecten maximus* [79].

In general, bivalve genomes are challenging to assemble because of their high levels of heterozygosity and repetitive DNA, particularly when short sequencing reads are being used. The problem of assembling highly polymorphic genomes with short read data is that standard genome assemblers often fail to recover the different haplotypes at heterozygous regions, reporting them as alternative contigs instead [80]. As a result, the assemblies of species with highly heterozygous genomes tend to be highly fragmented and have an artificially inflated size [81, 82]. Furthermore, bivalve genomic architecture has revealed a high prevalence of active transposable elements, which has made assembling accurate mollusc genomes difficult

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

due to high levels of repetition [71, 83]. Inbred individuals have been used for genome sequencing to try to mitigate the issues caused by high levels of heterozygosity [75]. However, this has not always resulted in the desired reduction in heterozygosity [71], and, could potentially reduce the range of genetic variation captured by the reference genome. Therefore, approaches to create phased, haplotype-aware genome assemblies are likely to be particularly useful for molluscs.

Third generation long-read sequencing technologies such as PacBio, Nanopore and Omni-C present a promising opportunity to improve bivalve genome assemblies, as they will allow resolution of complex regions [84]. Increased read length can potentially span the entirety of repetitive regions, which can prevent issues where the number of repeats cannot be resolved by short reads. Indeed the quality of mollusc genomes and gene annotation is already benefiting from this improved technology [79]. Importantly, the application of these technologies in combination with diploid-aware software can enable haplotype-phased assemblies [85, 86]. Resolving the diploid genome into haplotypes would provide a more accurate representation of the polymorphic landscape of bivalve genomes, facilitating downstream analysis. Mollusc genomes assembled with long read data currently include Snout Otter Clam *Lutraria rhynchaena* [87], black-shelled Pacific oyster *Crassostrea gigas* [88], the closely related *Crassostrea hongkongensis* [89], razor clam *Sinonovacula constricta* [90], blood clam *Scapharca broughtonii* [91], and Pacific oyster (Genbank Accession Number ASM1103280v1[92].

Other key genomic tools underpinning advances in selective breeding are related to high-throughput generation of genome-wide genetic markers, particularly single nucleotide polymorphisms (SNPs). This is typically performed using SNP arrays or genotyping by sequencing (GBS) approaches, each of which has advantages and disadvantages [93]. While

SNP arrays are more amenable to high-throughput genotyping of large numbers of individuals, they require significant up-front investment which tends to only be available for species with large production value and associated research communities. To date, in marine molluscs, SNP arrays are only available for Pacific and European flat oysters (Gutierrez et al. 2017, Qi et al. 2017). GBS approaches such as RAD-Seq or similar restriction enzyme-based approaches have been more broadly applied in molluscs to generate genome-wide SNP data [94], and this is partly due to its suitability for species for which very few genomic resources currently exist [95]. Both SNP arrays and GBS approaches are suitable for informing stock management, selection of animals to form base populations for selective breeding, and for assessment of the utility of genomic data in selective breeding programmes [20].

Genomics-enabled selective breeding

Genomic tools can improve genetic gain in molluscan selective breeding programmes via two main methods. The first is marker-assisted selection (MAS), whereby genetic markers linked to quantitative trait loci (QTL) affecting production traits are identified, and then subsequently genotyped to inform the choice of selection candidates. While this approach is likely to be successful for major-effect QTL, the majority of disease resistance traits tested in aquaculture species to date are underpinned by a polygenic architecture. The second approach is genomic selection, whereby the identification of specific QTL is less important, as genome-wide markers are used in the estimation of genomic breeding values (GEBVs) for selection candidates [96]. Initial studies testing genomic selection have shown promise in a multitude of aquaculture species (Zenger et al. 2018), including Pacific oysters [97, 98]. For example, genomic prediction of resistance to OsHV in Pacific oysters was substantially more accurate than using family or pedigree information alone [49]. Genomic selection can also be used in conjunction with so-called ‘sib-testing’ breeding designs, whereby full siblings of selection candidates are challenged with a pathogen, with mortalities and survivors being collected and

genotyped to inform on disease resistance. This allows accurate prediction of breeding values without collection of trait data on selection candidates themselves, and as such can help ensure biosecurity of the breeding nucleus as is commonplace in advanced finfish breeding programmes [48]. A major issue with genomic selection in marine mollusc breeding is that genotyping many individuals (selection candidates and reference populations) is expensive relative to the value of the animals. Therefore, approaches to reducing costs of genotyping are needed, such as genotype imputation whereby parents are genotyped at high density, and offspring at low density, then having genotypes imputed to high density [99].

Towards functional mechanisms underpinning genetic resistance in molluscs

While genomic selection does not require knowledge of the mechanisms underlying heritable variation in resistance to disease, there are several reasons why identifying the causative genes and variants is valuable in molluscs. Firstly, such genes and variants form potential targets for genome editing approaches to alter and fix disease resistance alleles in breeding populations. Secondly, they can help to inform alternative disease control strategies (e.g. immune priming, biosecurity). Thirdly, they can be used to improve genomic selection via applying differential weighting on functionally-relevant variants [100]. Application of SNP arrays and GBS for understanding the genetics of disease resistance are underway [25], and the typical first stage is identification of loci associated with the trait using genome-wide association studies (GWAS) [18, 93]. While disease resistance traits tend to be relatively polygenic, interesting QTL have been identified linked to genes in Pacific oyster that are relevant to viral adhesion and replication, release of viral particles, and the interferon gamma signalling pathway [25].

The regions identified by GWAS can provide targets for downstream studies investigating causative genes for disease resistance, but typically contain dozens of genes and thousands of variants. It is notoriously difficult to select candidate genes based on known literature, and

especially so in mollusc where knowledge of host response to infection is limited in comparison to better-studied farmed animals. Therefore, studies to shortlist potential functional genes and variants within the QTL region are key. These studies may take the form of a comparison of the host response to the pathogen in question between animals of alternate resistance genotypes, for example using RNA-Sequencing to assess gene expression, as has been performed for several diseases in oyster [101-103]. Overlap between differentially expressed genes and the QTL region may point to functional candidates, and QTL with cis-acting effects on gene expression (eQTL) can be used to further shortlist the candidate variants. Underpinning such research is the availability of high quality and well-annotated reference genomes. While the number of high quality mollusc genomes is increasingly rapidly, improvements in functional annotation of non-coding regions (e.g. promoters, enhancers) will greatly assist with identifying candidate functional variants [104, 105]. Finally, the rapid emergence of genome editing technologies is providing new opportunities to functionally validate such targets, for example via CRISPR/Cas gene knockout or modulation of gene expression.

Genome editing and transgenesis in Molluscs

The CRISPR/Cas9 system is a bacterial immune response system that has been repurposed to achieve highly accurate targeted editing of metazoan genomes via double strand break and repair in DNA [106]. CRISPR based technologies are developing rapidly and have demonstrated applications in gene knock-out, insertion of exogenous DNA and editing of specific nucleotides [106, 107]. There are few examples of CRISPR editing in molluscs to date (Table 2), but gene knockout via non-homologous end joining (described in Cong et al., 2013) has been successfully demonstrated in the freshwater snail *Lymnaea stagnalis*, the squid *Doryteuthis pealeii* and *C. gigas* [108-110]. Furthermore, exogenous DNA insertion via the homology directed repair (HDR) pathway has been demonstrated in the marine snail *Crepidula fornicata* [111]. These four studies are the only reported use of CRISPR in molluscs which

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

demonstrates that the CRISPR/Cas9 system can be successfully implemented, but knowledge of the optimal approaches for successful editing is limited. Cell lines are typically used at early stages of gene editing research, but there are no suitable cell lines for marine molluscs and use of primary culture has significant limitations [112].

Transgenesis, the process of introducing and expressing exogenous DNA from another organism, has also been successfully demonstrated in other molluscs (Table 2). Different approaches have been successfully demonstrated, including CRISPR/Cas9 HDR, plasmid and transposon mediated transfection (Table 2). Most of these studies have been for proof of concept, only focussing on expression of reporter proteins such as fluorescence. An increase in growth rate of abalone following introduction of fish growth hormone has been reported [113], but the mechanism of this response is yet to be clarified.

Table 2) Summary of previous studies involving molluscan transgenesis or editing

Reference	Species	Method	Insert	Reporter	Functional Promoter(s)	Efficiency of transgenesis (maximum)	Purpose
[114]	<i>Mulinia lateralis</i>	Pantropic retroviral vector/electroporation	LSRNL-(VSV-G)	β -galactosidase	RSV	33%	Proof of concept
[115]	<i>Crassostrea gigas</i>	Particle bombardment	pDrluc and pCMVL	Luciferase	Hsp70, CMV	0.1-0.5%	Proof of concept
[113]	<i>Haliotis diversicolor supertexta</i>	Electroporation of sperm	opAFP-GHc, opAFP-2000CAT	Genotyping by sequencing and southern blot analysis	SV40, AFP	20%	Increase growth rate
[116]	<i>Crassostrea gigas</i> cell cultures (heart/embryo)	Pantropic retroviral vector	LLRNL, LLRLL, LN-hsp70-lucL	Luciferase	RSV, Hsp70	0.5%	Proof of concept
[117]	<i>Crassostrea virginica</i>	Electroporation	Plasmid pS65T-C1	rsGFP	SV40, CMV	<1%	Proof of concept
[118]	<i>Mytilus galloprovincialis</i>	Electroporation/natural transfection	Plasmids pCMVlacZ and pMTbGH	Genotyping by sequencing	CMV, MT	Not reported (samples pooled for PCR)	Proof of concept
[119]	<i>Mytilus galloprovincialis</i> , <i>Mytilus chilensis</i> and <i>Chamelea gallina</i>	Natural transfection	p-GeneGrip	GFP	CMV	58.5 to 70.01%	Proof of concept
[120]	<i>Haliotis diversicolor supertexta</i>	Microinjection into testis	Plasmid pOBA-YPGHc	None	medaka promoter β -actin	20%	Increase growth rate
[121]	<i>Crassostrea gigas</i>	Electroporation	phsp-BiT-RFP-dsHOXCG1	RFP	Drosophila TRE, CMV	HSP70, 61%	Repressible lethality
[111]	<i>Crepidula fornicata</i>	Microinjection of Cas9 RNA, gRNA and template for Homology directed repair	β -catenin/ mCherry	mCherry	CMV	11%	Proof of concept
[122]	<i>Crassostrea gigas</i>	Electroporation of gametes/transposase	piggyBac Dual Promoter (with gGH)	GFP	CMV, EF1alpha	0.4%	Proof of concept
[109]	<i>Crassostrea gigas</i>	Microinjection of Cas9 RNA and gRNA targeting myostatin gene	N/A	Genotyping by sequencing	N/A	26.7%	Proof of concept
[106]	<i>Lymnaea stagnalis</i>	Microinjection of Cas9 RNA and gRNA targeting formin gene	N/A	Morphology and genotyping by sequencing	N/A	Up to 100%	Confirmation of formin gene function
[123]	<i>Haliotis discus hannai</i>	Microinjection of TALEN mRNA	N/A	Genotyping by sequencing	N/A	50%	Proof of concept
[124]	<i>Crassostrea gigas</i>	Microinjection of plasmids	Multiple plasmid fragments	EGFP	Endogenous elongation factor1- α	54%	Improve promotor
[125]	<i>Crassostrea virginica</i>	Chemical transfection of hemocytes	pCgVEGF-HA-IRES-GFP	GFP	CMV	0.13%	Proof of concept
[110]	<i>Doryteuthis pealeii</i>	Microinjection of Cas9 nuclease and CRISPR gRNAs	N/A	Visual inspection of natural pigmentation	N/A	>90%	Proof of concept

Abbreviations: CRISPR = clustered regularly interspaced short palindromic repeats, Cas = CRISPR associated protein, gRNA = guide RNA rsGFP = red shifted green fluorescent protein, GFP = green fluorescent protein, RFP = red fluorescent protein, RSV = rous sarcoma virus, Hsp70 = heat-shock protein 70, CMV = cytomegalovirus, SV40 = simian vacuolating virus 40, AFP = alpha-fetoprotein, MT = mouse metallothionein, TRE = tetracycline response element, EF1 = Human elongation factor-1, PCR = Polymerase chain reaction, TALEN = transcription activator-like effector nuclease

Future perspectives on genome editing in marine molluscs

The increasing demand for reliable production of marine molluscs makes mitigating impacts of disease essential. This can be achieved by maintaining and expanding breeding programmes to include the latest technologies to expedite genetic improvement. However, genome editing has potential to both improve selective breeding via the identification of functional variants impacting traits of interest, and via direct editing of mollusc broodstock for enhanced disease resistance in progeny. As described above, genome editing can be applied as an experimental tool in mollusc species to understand the functional basis of genes relating to disease resistance. This provides a means to improve understanding fundamental molluscan biology, in addition to the potential practical downstream benefits for aquaculture. For example, knock out of formin gene in freshwater snail *Lymnaea stagnalis* was used as confirmation of role in chirality of shell formation [108]. This can be applied to aquaculture relevant species focusing on genes relevant for disease resistance identified by genomic approaches [25, 102].

Genetic engineering approaches also have application beyond elucidating gene function in molluscs, which have certain amenable features of reproductive biology. Extreme fecundity can be used to overcome issues of poor transgenesis efficiency which can be a major problem for transgenesis / editing studies. Editing extremely large numbers will require mass delivery of CRISPR/Cas9 via electroporation or natural transfection, as opposed to microinjection of gametes (Table 2). Furthermore, high fecundity facilitates the use of high throughput disease challenge of edited larvae for rapid phenotyping of edits. Editing gametes may reduce the issue of mosaicism, as cells are edited prior to any nuclear division, but resulting edited embryos will be heterozygous for the target edit unless both eggs and sperm are edited. It may also be plausible to use sperm as the delivery vehicle for the CRISPR/Cas9 complex to edit the eggs after fertilisation, an approach shown to be successful in chickens [126]. The abundance of active transposable elements in molluscan genomes could be adapted to allow insertion of

exogenous DNA sequences, which has been successfully demonstrated in the pacific oyster to incorporate green fluorescing protein (GFP) [122]. Combined with the large number of gametes, low transgenesis and editing efficiency means there is a clear need for a high throughput screen to select for successfully edited / transgenic gametes / embryos. One possible option is to use a FACS (fluorescence activated cell sorting) based approach, where the cells (either gametes or embryos) contain a fluorescent protein, such as GFP. Incorporating fluorescent proteins has been successful in molluscs previously through CRISPR/Cas9 HDR, electroporation and natural transfection (Table 2). This approach would need to be optimised to account for issues such as delayed time until expression of fluorescent protein, and/or the relatively large size of embryos [127]. Alternatively, antibiotic selection using G418 has been demonstrated to be a potential approach for enrichment of transfected embryos in *Crassostrea virginica* [117].

While early studies in aquaculture species have focussed on editing single genes [128], there is potential in highly multiplexed editing and screening experiments. Genome wide CRISPR knock-out (GeCKO) and synergistic activation mediator (SAM) are approaches to either disrupt or upregulate a single specific gene in one cell within a population of thousands of uniquely edited cells [129-131]. These screens involve the design of a library of tens of thousands of unique guide RNAs, typically aiming to target every gene in the organism. These gRNAs are packaged into a lentivirus vector, and transduced into cells (typically cell lines constitutively expressing Cas9) at a dose aiming for approximately one gRNA integration per cell. The edited cell line is then screened (e.g., using a pathogen challenge) and the selected cells (e.g. surviving cells or those expressing a fluorescent marker) are sequenced. The enrichment or depletion of gRNAs thereby informs on the putative role of the target genes in the phenotype under investigation. Such approaches could potentially be extended to in vivo studies in molluscs, with early life larval screens replacing the cell line. An effective high

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

throughput screening system combined with mass spawning of molluscs could enable development of a high throughput whole-organism genetic editing system. Larval disease challenges can be included as a high-throughput approach to identify disease resistance candidate genes for downstream study, or editing for DNA associated with resistance [25] for commercial production.

Finally, once putative disease resistance alleles have been detected or created, it may be plausible to use genome editing of commercial broodstock to breed disease resistant molluscs for aquaculture. This approach would need to be integrated within a well-managed breeding programme to ensure that there was appropriate maintenance of genetic diversity, and focus on multiple traits. Furthermore, substantial consideration would need to be given to both the practicalities of commercial-scale application, and the public perception and regulatory landscape surrounding genetic engineering and genome editing. An advantage of using CRISPR/Cas editing (rather than introduction of exogenous DNA) is that resulting animals are not transgenic (although they may be still be classified as genetically modified), and have no foreign DNA in their genomes. This is a key consideration for animals that are intended for possible production and consumption, as previously developed transgenic animals intended for human consumption (e.g. AquAdvantage salmon from AquaBounty™) has been subject to a lengthy and difficult regulatory progress [132]. However, the legal and regulatory landscape is rapidly evolving and varies substantially according to geographical location. It is important that engagement with regulators, public, and other stakeholders relating to the benefits and risks of such approaches goes alongside the technical developments and their application to mollusc aquaculture.

Conclusions

Disease resistance in marine molluscs has been demonstrated to be heritable and amenable to improvement via selective breeding approaches. As uptake of breeding technology increases across mollusc aquaculture, genetic improvement could be expedited by the use of the rapidly expanding genomic toolbox, via cost-effective applications of genomic selection. Furthermore, as genome editing and transgenesis approaches develop, they will facilitate identification of functional disease resistance alleles, which will both improve our understanding of disease biology, and provide potential novel avenues for commercial application. These approaches together hold substantial potential to produce molluscs with resistance to existing and future pathogens for aquaculture.

Acknowledgements

This work was supported by the Biotechnology and Biological Sciences Research Council (BBSRC) (grant number BB/M010996/1), BBSRC Institute Strategic Programme Grants (BBS/E/D/20002172 and BBS/E/D/30002275) and Centre for Environment, Fisheries and Aquaculture Science (Cefas) Seedcorn project DP901W.

References

- 1 FAO. 2019 The State of the World's Aquatic Genetic Resources for Food and Agriculture. FAO Commission on Genetic Resources for Food and Agriculture assessments. Rome.,
- 2 FAO. 2018 The State of World Fisheries and Aquaculture 2018 - Meeting the sustainable development goals. Rome. .
- 3 Froehlich, H. E., Runge, C. A., Gentry, R. R., Gaines, S. D., Halpern, B. S. 2018 Comparative terrestrial feed and land use of an aquaculture-dominant world. **115**, 5295-5300. (doi:10.1073/pnas.1801692115)
- 4 Vidal, E. A., Villanueva, R., Andrade, J. P., Gleadall, I. G., Iglesias, J., Koueta, N., Rosas, C., Segawa, S., Grasse, B., Franco-Santos, R. M., *et al.* 2014 Cephalopod culture: current status of main biological models and research priorities. *Advances in marine biology*. **67**, 1-98. (doi:10.1016/b978-0-12-800287-2.00001-9)
- 5 O'Brien, C. E., Roumbedakis, K., Winkelmann, I. E. 2018 The Current State of Cephalopod Science and Perspectives on the Most Critical Challenges Ahead From Three Early-Career Researchers. **9**, (doi:10.3389/fphys.2018.00700)

- 6 Mao, Y., Lin, F., Fang, J., Fang, J., Li, J., Du, M. 2019 Bivalve Production in China. In *Goods and Services of Marine Bivalves*. (Editors: A. C. Smaal, J. G. Ferreira, J. Grant, J. K. Petersen, Ø. Strand), pp. 51-72. Cham: Springer International Publishing.
- 7 FAO. 2016 The State of World Fisheries and Aquaculture 2016. Contributing to food security and nutrition for all. Rome.
- 8 Arzul, I., Corbeil, S., Morga, B., Renault, T. 2017 Viruses infecting marine molluscs. *J. Invertebr. Pathol.* **147**, 118-135. (doi:10.1016/j.jip.2017.01.009)
- 9 Lafferty, K. D., Harvell, C. D., Conrad, J. M., Friedman, C. S., Kent, M. L., Kuris, A. M., Powell, E. N., Rondeau, D., Saksida, S. M. 2015 Infectious Diseases Affect Marine Fisheries and Aquaculture Economics. In *Annual Review of Marine Science, Vol 7*. (Editors C. A. Carlson, S. J. Giovannoni), pp. 471-496. Palo Alto: Annual Reviews.
- 10 Bower, S. M., McGladdery, S. E., Price, I. M. 1994 Synopsis of infectious diseases and parasites of commercially exploited shellfish. *Annual Review of Fish Diseases*. **4**, 1-199. (doi:10.1016/0959-8030(94)90028-0)
- 11 Pernet, F., Lupo, C., Bacher, C., Whittington, R. J. 2016 Infectious diseases in oyster aquaculture require a new integrated approach. **371**, 20150213. (doi:10.1098/rstb.2015.0213)
- 12 Tan, K., Zhang, H., Zheng, H. 2020 Selective breeding of edible bivalves and its implication of global climate change. *n/a*, (doi:10.1111/raq.12458)
- 13 Yáñez, J. M., Houston, R. D., Newman, S. 2014 Genetics and genomics of disease resistance in salmonid species. *Front. Genet.* **5**, 415-415. (doi:10.3389/fgene.2014.00415)
- 14 Gjedrem, T. 2015 Disease Resistant Fish and Shellfish Are within Reach: A Review. *J. Mar. Sci. Eng.* **3**, 146-153. (doi:10.3390/jmse3010146)
- 15 Houston, R. D. 2017 Future directions in breeding for disease resistance in aquaculture species. *Revista Brasileira De Zootecnia-Brazilian Journal of Animal Science*. **46**, 545-551. (doi:10.1590/s1806-92902017000600010)
- 16 You, X., Shan, X., Shi, Q. 2020 Research advances in the genomics and applications for molecular breeding of aquaculture animals. *Aquaculture*. **526**, 735357. (doi:10.1016/j.aquaculture.2020.735357)
- 17 Wang, L., Qiu, L., Zhou, Z., Song, L. 2013 Research progress on the mollusc immunity in China. *Dev Comp Immunol.* **39**, 2-10. (doi:10.1016/j.dci.2012.06.014)
- 18 Hollenbeck, C. M., Johnston, I. A. 2018 Genomic tools and selective breeding in molluscs. *Front. Genet.* **9**, (doi:10.3389/fgene.2018.00253)
- 19 Gjedrem, T., Rye, M. 2018 Selection response in fish and shellfish: a review. *Reviews in Aquaculture*. **10**, 168-179. (doi:10.1111/raq.12154)
- 20 Houston, R. D., Bean, T. P., Macqueen, D. J., Gundappa, M. K., Jin, Y. H., Jenkins, T. L., Selly, S. L. C., Martin, S. A. M., Stevens, J. R., Santos, E. M., *et al.* 2020 Harnessing genomics to fast-track genetic improvement in aquaculture. *Nature Reviews Genetics*. (doi:10.1038/s41576-020-0227-y)
- 21 Azema, P., Maurouard, E., Lamy, J. B., Degremont, L. 2017 The use of size and growing height to improve *Crassostrea gigas* farming and breeding techniques against OsHV-1. *Aquaculture*. **471**, 121-129. (doi:10.1016/j.aquaculture.2017.01.011)
- 22 Camara, M. D., Symonds, J. E. 2014 Genetic improvement of New Zealand aquaculture species: programmes, progress and prospects. *N. Z. J. Mar. Freshw. Res.* **48**, 466-491. (doi:10.1080/00288330.2014.932291)
- 23 Degremont, L., Garcia, C., Allen, S. K. 2015 Genetic improvement for disease resistance in oysters: A review. *J. Invertebr. Pathol.* **131**, 226-241. (doi:10.1016/j.jip.2015.05.010)
- 24 Degremont, L., Lamy, J. B., Pepin, J. F., Travers, M. A., Renault, T. 2015 New Insight for the Genetic Evaluation of Resistance to Ostreid Herpesvirus Infection, a Worldwide Disease, in *Crassostrea gigas*. *Plos One*. **10**, (doi:10.1371/journal.pone.0127917)
- 25 Gutierrez, A. P., Bean, T. P., Hooper, C., Stenton, C. A., Sanders, M. B., Paley, R. K., Rastas, P., Bryrom, M., Matika, O., Houston, R. D. 2018 A Genome-Wide Association Study for Host Resistance

- to Ostreid Herpesvirus in Pacific Oysters (*Crassostrea gigas*). *G3-Genes Genomes Genet.* **8**, 1273-1280. (doi:10.1534/g3.118.200113)
- 26 Lough, G., Kyriazakis, I., Bergmann, S., Lengeling, A., Doeschl-Wilson, A. B. 2015 Health trajectories reveal the dynamic contributions of host genetic resistance and tolerance to infection outcome. *Proceedings. Biological sciences.* **282**, (doi:10.1098/rspb.2015.2151)
- 27 Brokordt, K., González, R., Farías, W., Winkler, F. E., Lohrmann, K. B. 2017 First insight into the heritable variation of the resistance to infection with the bacteria causing the withering syndrome disease in *Haliotis rufescens* abalone. *J. Invertebr. Pathol.* **150**, 15-20. (doi:10.1016/j.jip.2017.08.014)
- 28 Stear, M. J., Bishop, S. C., Mallard, B. A., Raadsma, H. 2001 The sustainability, feasibility and desirability of breeding livestock for disease resistance. *Research in Veterinary Science.* **71**, 1-7. (doi:10.1053/rvsc.2001.0496)
- 29 Saura, M., Carabaño, M. J., Fernández, A., Cabaleiro, S., Doeschl-Wilson, A. B., Anacleto, O., Maroso, F., Millán, A., Hermida, M., Fernández, C., *et al.* 2019 Disentangling Genetic Variation for Resistance and Endurance to Scuticociliatosis in Turbot Using Pedigree and Genomic Information. **10**, (doi:10.3389/fgene.2019.00539)
- 30 Loker, E. S., Adema, C. M., Zhang, S.-M., Kepler, T. B. 2004 Invertebrate immune systems--not homogeneous, not simple, not well understood. *Immunological reviews.* **198**, 10-24. doi: (doi:10.1111/j.0105-2896.2004.0117)
- 31 Allam, B., Raftos, D. 2015 Immune responses to infectious diseases in bivalves. *J. Invertebr. Pathol.* **131**, 121-136. (doi:10.1016/j.jip.2015.05.005)
- 32 Green, T. J., Speck, P. 2018 Antiviral Defense and Innate Immune Memory in the Oyster. *Viruses.* **10**, (doi:10.3390/v10030133)
- 33 Lafont, M., Vergnes, A., Vidal-Dupiol, J., de Lorigeril, J., Gueguen, Y., Haffner, P., Petton, B., Chaparro, C., Barrachina, C., Destoumieux-Garzon, D., *et al.* 2020 A Sustained Immune Response Supports Long-Term Antiviral Immune Priming in the Pacific Oyster, *Crassostrea gigas* **11**, e02777-02719. (doi:10.1128/mBio.02777-19)
- 34 Camara, M. D., Yen, S., Kaspar, H. F., Kesarcodi-Watson, A., King, N., Jeffs, A. G., Tremblay, L. A. 2017 Assessment of heat shock and laboratory virus challenges to selectively breed for ostreid herpesvirus 1 (OsHV-1) resistance in the Pacific oyster, *Crassostrea gigas*. *Aquaculture.* **469**, 50-58. (doi:10.1016/j.aquaculture.2016.11.031)
- 35 Nicolas, J. L., Comps, M., Cochennec, N. 1992 Herpes-like virus infecting Pacific-oyster larvae, *Crassostrea gigas*. *B Eur Assoc Fish Pat.* **12**, 11-13.
- 36 Segarra, A., Pepin, J. F., Arzul, I., Morga, B., Fauray, N., Renault, T. 2010 Detection and description of a particular Ostreid herpesvirus 1 genotype associated with massive mortality outbreaks of Pacific oysters, *Crassostrea gigas*, in France in 2008. *Virus Res.* **153**, 92-99. (doi:10.1016/j.virusres.2010.07.011)
- 37 Jenkins, C., Hick, P., Gabor, M., Spiers, Z., Fell, S. A., Gu, X. N., Read, A., Go, J., Dove, M., O'Connor, W., *et al.* 2013 Identification and characterisation of an ostreid herpesvirus-1 microvariant (OsHV-1 mu-var) in *Crassostrea gigas* (Pacific oysters) in Australia. *Dis. Aquat. Org.* **105**, 109-126. (doi:10.3354/dao02623)
- 38 Doonan, I. J., Cranfield, H. J., Michael, K. P. 1994 Catastrophic reduction of the oyster, *Tiostrea chilensis* (Bivalvia: Ostreidae), in Foveaux Strait, New Zealand, due to infestation by the protistan *Bonamia* sp. *N. Z. J. Mar. Freshw. Res.* **28**, 335-344. (doi:10.1080/00288330.1994.9516623)
- 39 Carrasco, N., Villalba, A., Andree, K. B., Engelsma, M. Y., Lacuesta, B., Ramilo, A., Gairín, I., Furones, M. D. 2012 *Bonamia exitiosa* (Haplosporidia) observed infecting the European flat oyster *Ostrea edulis* cultured on the Spanish Mediterranean coast. *J. Invertebr. Pathol.* **110**, 307-313. (doi:10.1016/j.jip.2012.03.015)
- 40 Ford, S. E., Smolowitz, R. J. M. B. 2007 Infection dynamics of an oyster parasite in its newly expanded range. **151**, 119-133. (doi:10.1007/s00227-006-0454-6)

- 41 Ford, S. E., Haskin, H. H. 1982 History and epizootiology of *Haplosporidium nelsoni* (MSX), an oyster pathogen in Delaware Bay, 1957–1980. *J. Invertebr. Pathol.* **40**, 118–141. (doi:10.1016/0022-2011(82)90043-X)
- 42 Divilov, K., Schoolfield, B., Morga, B., Dégremont, L., Burge, C. A., Mancilla Cortez, D., Friedman, C. S., Fleener, G. B., Dumbauld, B. R., Langdon, C. 2019 First evaluation of resistance to both a California OsHV-1 variant and a French OsHV-1 microvariant in Pacific oysters. *BMC Genetics*. **20**, 96. (doi:10.1186/s12863-019-0791-3)
- 43 Dégremont, L., Azéma, P., Maurouard, E., Travers, M.-A. 2020 Enhancing resistance to *Vibrio aestuarianus* in *Crassostrea gigas* by selection. *Aquaculture*. **526**, 735429. (doi:10.1016/j.aquaculture.2020.735429)
- 44 Liu, B., Tan, C., Zhang, D., Chen, M., Niu, Z., Huang, G., Li, Y., Zhang, T., Yu, D. 2017 Genetic parameters of growth and resistance to *Polydora ciliata* in the pearl oyster *Pinctada fucata*. **48**, 2039–2046. (doi:10.1111/are.13036)
- 45 Azema, P., Lamy, J. B., Boudry, P., Renault, T., Travers, M. A., Degremont, L. 2017 Genetic parameters of resistance to *Vibrio aestuarianus*, and OsHV-1 infections in the Pacific oyster, *Crassostrea gigas*, at three different life stages. *Genet. Sel. Evol.* **49**, 16. (doi:10.1186/s12711-017-0297-2)
- 46 Hayes, B. J., Lewin, H. A., Goddard, M. E. 2013 The future of livestock breeding: genomic selection for efficiency, reduced emissions intensity, and adaptation. *Trends in Genetics*. **29**, 206–214. (doi:10.1016/j.tig.2012.11.009)
- 47 Dégremont, L., Nourry, M., Maurouard, E. 2015 Mass selection for survival and resistance to OsHV-1 infection in *Crassostrea gigas* spat in field conditions: response to selection after four generations. *Aquaculture*. **446**, 111–121. (doi:10.1016/j.aquaculture.2015.04.029)
- 48 Zenger, K. R., Khatkar, M. S., Jones, D. B., Khalilisamani, N., Jerry, D. R., Raadsma, H. W. 2018 Genomic Selection in Aquaculture: Application, Limitations and Opportunities With Special Reference to Marine Shrimp and Pearl Oysters. *Front Genet.* **9**, 693. (10.3389/fgene.2018.00693)
- 49 Gutierrez, A. P., Symonds, J., King, N., Steiner, K., Bean, T. P., Houston, R. D. 2020 Potential of genomic selection for improvement of resistance to ostreid herpesvirus in Pacific oyster (*Crassostrea gigas*). **51**, 249–257. (doi:10.1111/age.12909)
- 50 Hedgecock, D., McGoldrick, D. J., Bayne, B. L. 1995 Hybrid vigor in Pacific oysters: an experimental approach using crosses among inbred lines. *Aquaculture*. **137**, 285–298. (doi:10.1016/0044-8486(95)01105-6)
- 51 Tan, K. S., Zhang, H., Liu, H., Cheng, D., Ye, T., Ma, H., Li, S., Zheng, H. 2019 Enhancing lipid nutritional quality of oysters by hybridization between *Crassostrea gigas* and *C. angulata*. **50**, 3776–3782. (doi:10.1111/are.14340)
- 52 Hedgecock, D., Davis, J. P. 2007 Heterosis for yield and crossbreeding of the Pacific oyster *Crassostrea gigas*. *Aquaculture*. **272**, S17–S29. (doi:10.1016/j.aquaculture.2007.07.226)
- 53 Li, J., Wang, M., Fang, J., Liu, X., Xue, S., Mao, Y., Liu, G. 2018 A comparison of offspring growth and survival among a wild and a selected strain of the Pacific abalone (*Haliotis discus hannai*) and their hybrids. *Aquaculture*. **495**, 721–725. (doi:10.1016/j.aquaculture.2018.06.071)
- 54 Kong, L., Song, S., Li, Q. 2017 The effect of interstrain hybridization on the production performance in the Pacific oyster *Crassostrea gigas*. *Aquaculture*. **472**, 44–49. (doi:10.1016/j.aquaculture.2016.07.018)
- 55 Zhang, Y., Zhang, Y., Li, J., Xiao, S., Xiang, Z., Wang, Z., Yan, X., Yu, Z. 2016 Artificial interspecific backcrosses between the hybrid of female *Crassostrea hongkongensis* × male *C. gigas* and the two parental species. *Aquaculture*. **450**, 95–101. (doi:10.1016/j.aquaculture.2015.07.013)
- 56 Iversen, M. W., Nordbø, Ø., Gjerlaug-Enger, E., Grindflek, E., Lopes, M. S., Meuwissen, T. 2019 Effects of heterozygosity on performance of purebred and crossbred pigs. *Genet. Sel. Evol.* **51**, 8. (doi:10.1186/s12711-019-0450-1)
- 57 de Lorgeril, J., Lucasson, A., Petton, B., Toulza, E., Montagnani, C., Clerissi, C., Vidal-Dupiol, J., Chaparro, C., Galinier, R., Escoubas, J.-M., et al. 2018 Immune-suppression by OsHV-1 viral infection

- causes fatal bacteraemia in Pacific oysters. *Nature Communications*. **9**, 4215. (doi:10.1038/s41467-018-06659-3)
- 58 Azéma, P., Lamy, J.-B., Boudry, P., Renault, T., Travers, M.-A., Dégremon, L. 2017 Genetic parameters of resistance to *Vibrio aestuarianus*, and OsHV-1 infections in the Pacific oyster, *Crassostrea gigas*, at three different life stages. *Genet. Sel. Evol.* **49**, 23. (doi:10.1186/s12711-017-0297-2)
- 59 Divilov, K., Schoolfield, B., Morga, B., Dégremon, L., Burge, C. A., Mancilla Cortez, D., Friedman, C. S., Fleener, G. B., Dumbauld, B. R., Langdon, C. 2019 First evaluation of resistance to both a California OsHV-1 variant and a French OsHV-1 microvariant in Pacific oysters. *BMC genetics*. **20**, 96-96. (doi:10.1186/s12863-019-0791-3)
- 60 Kube, P., Cunningham, M., Dominik, S., Parkinson, S., Finn, B., Henshall, J., Bennett, R., Hamilton, M. 2011 *Enhancement of the Pacific Oyster Selective Breeding Program*.
- 61 Symonds, J. E., Clarke, S. M., King, N., Walker, S. P., Blanchard, B., Sutherland, D., Roberts, R., Preece, M. A., Tate, M., Buxton, P., *et al.* 2019 Developing Successful Breeding Programs for New Zealand Aquaculture: A Perspective on Progress and Future Genomic Opportunities. *Front Genet.* **10**, 27. (doi:10.3389/fgene.2019.00027)
- 62 Frank-Lawale, A., Jr., S. K. A., Dégremon, L. 2014 Breeding and Domestication of Eastern Oyster *Crassostrea virginica* Lines for Culture in the Mid-Atlantic, Usa: Line Development and Mass Selection for Disease Resistance. **33**, 153-165, 113. (doi:10.2983/035.033.0115)
- 63 Proestou, D. A., Vinyard, B. T., Corbett, R. J., Piesz, J., Allen, S. K., Small, J. M., Li, C., Liu, M., DeBrosse, G., Guo, X., *et al.* 2016 Performance of selectively-bred lines of eastern oyster, *Crassostrea virginica*, across eastern US estuaries. *Aquaculture*. **464**, 17-27. (doi:10.1016/j.aquaculture.2016.06.012)
- 64 Casas, S., Walton, W., Chaplin, G., Rikard, S., Supan, J., La Peyre, J. 2017 Performance of oysters selected for dermo resistance compared to wild oysters in northern Gulf of Mexico estuaries. *Aquaculture Environment Interactions*. **9**, 169-180. (doi:org/10.3354/aei00222)
- 65 Proestou, D. A., Corbett, R. J., Ben-Horin, T., Small, J. M., Allen Jr, S. K. 2019 Defining Dermo resistance phenotypes in an eastern oyster breeding population. **50**, 2142-2154. (doi:10.1111/are.14095)
- 66 Siva, V. S., Yang, C., Yang, J., Wang, L., Wang, L., Zhou, Z., Qiu, L., Song, L. 2012 Association of CflGBP gene polymorphism with disease susceptibility/resistance of Zhikong scallop (*Chlamys farreri*) to *Listonella anguillarum*. *Fish Shellfish Immunol.* **32**, 1117-1123. (doi:10.1016/j.fsi.2012.03.017)
- 67 Nie, Q., Yue, X., Liu, B. 2015 Development of *Vibrio* spp. infection resistance related SNP markers using multiplex SNaPshot genotyping method in the clam *Meretrix meretrix*. *Fish Shellfish Immunol.* **43**, 469-476. (doi:10.1016/j.fsi.2015.01.030)
- 68 Vaibhav, V., Thompson, E. L., Raftos, D. A., Haynes, P. A. 2018 Potential protein biomarkers of QX disease resistance in selectively bred Sydney Rock Oysters. *Aquaculture*. **495**, 144-152. (doi:10.1016/j.aquaculture.2018.05.035)
- 69 Vaibhav, V., Lepretre, M., Thompson, E., Raftos, D., Haynes, P. A. 2016 Biomarkers of Winter Mortality resistance in selectively bred Sydney rock oysters (*Saccostrea glomerata*). *Aquaculture*. **465**, 323-329. (doi:10.1016/j.aquaculture.2016.09.006)
- 70 Berg, P. 2006 Origins of the human genome project: why sequence the human genome when 96% of it is junk? *American journal of human genetics*. **79**, 603-605. (doi:10.1086/507688)
- 71 Zhang, G., Fang, X., Guo, X., Li, L., Luo, R., Xu, F., Yang, P., Zhang, L., Wang, X., Qi, H., *et al.* 2012 The oyster genome reveals stress adaptation and complexity of shell formation. *Nature*. **490**, 49-54. (doi:10.1038/nature11413)
- 72 Takeuchi, T., Kawashima, T., Koyanagi, R., Gyoja, F., Tanaka, M., Ikuta, T., Shoguchi, E., Fujiwara, M., Shinzato, C., Hisata, K., *et al.* 2012 Draft genome of the pearl oyster *Pinctada fucata*: a platform for understanding bivalve biology. *DNA research : an international journal for rapid publication of reports on genes and genomes*. **19**, 117-130. (doi:10.1093/dnares/dss005)

- 73 Takeuchi, T., Koyanagi, R., Gyoja, F., Kanda, M., Hisata, K., Fujie, M., Goto, H., Yamasaki, S., Nagai, K., Morino, Y., *et al.* 2016 Bivalve-specific gene expansion in the pearl oyster genome: implications of adaptation to a sessile lifestyle. *Zoological Letters*. **2**, 3. (doi:10.1186/s40851-016-0039-2)
- 74 Du, X., Fan, G., Jiao, Y., Zhang, H., Guo, X., Huang, R., Zheng, Z., Bian, C., Deng, Y., Wang, Q., *et al.* 2017 The pearl oyster *Pinctada fucata martensii* genome and multi-omic analyses provide insights into biomineralization. *GigaScience*. **6**, 1-12. (doi:10.1093/gigascience/gix059)
- 75 Wang, S., Zhang, J., Jiao, W., Li, J., Xun, X., Sun, Y., Guo, X., Huan, P., Dong, B., Zhang, L., *et al.* 2017 Scallop genome provides insights into evolution of bilaterian karyotype and development. *Nature Ecology & Evolution*. **1**, 0120. (doi:10.1038/s41559-017-0120)
- 76 Sun, J., Zhang, Y., Xu, T., Zhang, Y., Mu, H., Zhang, Y., Lan, Y., Fields, C. J., Hui, J. H. L., Zhang, W., *et al.* 2017 Adaptation to deep-sea chemosynthetic environments as revealed by mussel genomes. *Nature Ecology & Evolution*. **1**, 0121. (doi:10.1038/s41559-017-0121)
- 77 Murgarella, M., Puiu, D., Novoa, B., Figueras, A., Posada, D., Canchaya, C. 2016 A First Insight into the Genome of the Filter-Feeder Mussel *Mytilus galloprovincialis*. *Plos One*. **11**, e0151561. (doi:10.1371/journal.pone.0151561)
- 78 Elizur, A., Zhao, M., Subramanian, S., Suwansa-ard, S., Powell, D., O'Connor, W., Raftos, D. 2018 The genome of the oyster *Saccostrea* offers insight into the environmental resilience of bivalves. *DNA Research*. **25**, 655-665. (doi:10.1093/dnares/dsy032)
- 79 Kenny, N. J., McCarthy, S. A., Dudchenko, O., James, K., Betteridge, E., Corton, C., Dolucan, J., Mead, D., Oliver, K., Omer, A. D., *et al.* 2020 The gene-rich genome of the scallop *Pecten maximus*. *GigaScience*. **9**, (doi:10.1093/gigascience/giaa037)
- 80 Pruszcz, L. P., Gabaldón, T. 2016 Redundans: an assembly pipeline for highly heterozygous genomes. *Nucleic acids research*. **44**, e113-e113. (doi:10.1093/nar/gkw294)
- 81 Small, K. S., Brudno, M., Hill, M. M., Sidow, A. 2007 A haplome alignment and reference sequence of the highly polymorphic *Ciona savignyi* genome. *Genome biology*. **8**, R41. (doi:10.1186/gb-2007-8-3-r41)
- 82 Pruszcz, L. P., Németh, T., Gácsér, A., Gabaldón, T. 2014 Genome comparison of *Candida orthopsilosis* clinical strains reveals the existence of hybrids between two distinct subspecies. *Genome biology and evolution*. **6**, 1069-1078. (doi:10.1093/gbe/evu082)
- 83 Puzakov, M. V., Puzakova, L. V., Cheresiz, S. V. 2018 An Analysis of IS630/Tc1/mariner Transposons in the Genome of a Pacific Oyster, *Crassostrea gigas*. *Journal of Molecular Evolution*. **86**, 566-580. (doi:10.1007/s00239-018-9868-2)
- 84 Gurdasani, D., Sandhu, M. S., Porter, T., Pollard, M. O., Mentzer, A. J. 2018 Long reads: their purpose and place. *Human Molecular Genetics*. **27**, R234-R241. (doi:10.1093/hmg/ddy177)
- 85 Kronenberg, Z. N., Rhie, A., Koren, S., Concepcion, G. T., Peluso, P., Munson, K. M., Hiendleder, S., Fedrigo, O., Jarvis, E. D., Phillippy, A. M., *et al.* 2019 Extended haplotype phasing of de novo genome assemblies with FALCON-Phase. 327064. (doi:10.1101/327064)
- 86 Ananthasayanam, S., Kothandaraman, H., Nayee, N., Saha, S., Baghel, D. S., Gopalakrishnan, K., Peddamma, S., Singh, R. B., Schatz, M. 2019 First near complete haplotype phased genome assembly of River buffalo *Bubalus bubalis*. 618785. (doi:10.1101/618785)
- 87 Thai, B. T., Lee, Y. P., Gan, H. M., Austin, C. M., Croft, L. J., Trieu, T. A., Tan, M. H. 2019 Whole Genome Assembly of the Snout Otter Clam, *Lutraria rhynchaena*, Using Nanopore and Illumina Data, Benchmarked Against Bivalve Genome Assemblies. **10**, (doi:10.3389/fgene.2019.01158)
- 88 Wang, X., Xu, W., Wei, L., Zhu, C., He, C., Song, H., Cai, Z., Yu, W., Jiang, Q., Li, L., *et al.* 2019 Nanopore Sequencing and De Novo Assembly of a Black-Shelled Pacific Oyster (*Crassostrea gigas*) Genome. *Front Genet*. **10**, 1211. (doi:10.3389/fgene.2019.01211)

- 89 Peng, J., Li, Q., Xu, L., Wei, P., He, P., Zhang, X., Zhang, L., Guan, J., Zhang, X., Lin, Y., *et al.* 2020 Chromosome-level analysis of the *Crassostrea hongkongensis* genome reveals extensive duplication of immune-related genes in bivalves. *n/a*, (doi:10.1111/1755-0998.13157)
- 90 Ran, Z., Li, Z., Yan, X., Liao, K., Kong, F., Zhang, L., Cao, J., Zhou, C., Zhu, P., He, S., *et al.* 2019 Chromosome-level genome assembly of the razor clam *Sinonovacula constricta* (Lamarck, 1818). **19**, 1647-1658. (doi:10.1111/1755-0998.13086)
- 91 Bai, C.-M., Xin, L.-S., Rosani, U., Wu, B., Wang, Q.-C., Duan, X.-K., Liu, Z.-H., Wang, C.-M. 2019 Chromosomal-level assembly of the blood clam, *Scapharca (Anadara) broughtonii*, using long sequence reads and Hi-C. *GigaScience*. **8**, (doi:10.1093/gigascience/giz067)
- 92 Peñaloza, C., Gutierrez, A. P., Eory, L., Wang, S., Guo, X., Archibald, A. L., Bean, T. P., Houston, R. D. 2020 A chromosome-level genome assembly for the Pacific oyster (*Crassostrea gigas*). 2020.2009.2025.313494. (doi:10.1101/2020.09.25.313494)
- 93 Robledo, D., Palaiokostas, C., Bargelloni, L., Martinez, P., Houston, R. 2018 Applications of genotyping by sequencing in aquaculture breeding and genetics. *Reviews in Aquaculture*. **10**, 670-682. (doi:10.1111/raq.12193)
- 94 Gomes-dos-Santos, A., Lopes-Lima, M., Castro, L. F. C., Froufe, E. 2020 Molluscan genomics: the road so far and the way forward. *Hydrobiologia*. **847**, 1705-1726. (doi:10.1007/s10750-019-04111-1)
- 95 Díaz-Arce, N., Rodríguez-Ezpeleta, N. 2019 Selecting RAD-Seq Data Analysis Parameters for Population Genetics: The More the Better? **10**, (doi:10.3389/fgene.2019.00533)
- 96 Meuwissen, T. H., Hayes, B. J., Goddard, M. E. 2001 Prediction of total genetic value using genome-wide dense marker maps. *Genetics*. **157**, 1819-1829.
- 97 Gutierrez, A. P., Symonds, J., King, N., Steiner, K., Bean, T. P., Houston, R. D. 2019 Potential of genomic selection for improvement of resistance to Ostreid Herpes virus in Pacific oyster *Crassostrea gigas*. 754473. (doi:10.1101/754473)
- 98 Gutierrez, A. P., Matika, O., Bean, T. P., Houston, R. D. 2018 Genomic Selection for Growth Traits in Pacific Oyster (*Crassostrea gigas*): Potential of Low-Density Marker Panels for Breeding Value Prediction. *Front. Genet.* **9**, 391-391. (doi:10.3389/fgene.2018.00391)
- 99 Tsairidou, S., Hamilton, A., Robledo, D., Bron, J. E., Houston, R. D. 2020 Optimizing Low-Cost Genotyping and Imputation Strategies for Genomic Selection in Atlantic Salmon. **10**, 581-590. (doi:10.1534/g3.119.400800)
- 100 MacLeod, I. M., Bowman, P. J., Vander Jagt, C. J., Haile-Mariam, M., Kemper, K. E., Chamberlain, A. J., Schrooten, C., Hayes, B. J., Goddard, M. E. 2016 Exploiting biological priors and sequence variants enhances QTL discovery and genomic prediction of complex traits. *BMC Genomics*. **17**, 144. (doi:10.1186/s12864-016-2443-6)
- 101 McDowell, I. C., Nikapitiya, C., Aguiar, D., Lane, C. E., Istrail, S., Gomez-Chiarri, M. 2014 Transcriptome of American Oysters, *Crassostrea virginica*, in Response to Bacterial Challenge: Insights into Potential Mechanisms of Disease Resistance. *Plos One*. **9**, e105097. (doi:10.1371/journal.pone.0105097)
- 102 de Lorgeril, J., Petton, B., Lucasson, A., Perez, V., Stenger, P.-L., Dégremont, L., Montagnani, C., Escoubas, J.-M., Haffner, P., Allienne, J.-F., *et al.* 2020 Differential basal expression of immune genes confers *Crassostrea gigas* resistance to Pacific oyster mortality syndrome. *BMC Genomics*. **21**, 63. (doi:10.1186/s12864-020-6471-x)
- 103 Proestou, D. A., Sullivan, M. E. 2020 Variation in global transcriptomic response to *Perkinsus marinus* infection among eastern oyster families highlights potential mechanisms of disease resistance. *Fish Shellfish Immunol.* **96**, 141-151. (doi:10.1016/j.fsi.2019.12.001)
- 104 Andersson, L., Archibald, A. L., Bottema, C. D., Brauning, R., Burgess, S. C., Burt, D. W., Casas, E., Cheng, H. H., Clarke, L., Couldrey, C., *et al.* 2015 Coordinated international action to accelerate genome-to-phenome with FAANG, the Functional Annotation of Animal Genomes project. *Genome Biology*. **16**, 57. (doi:10.1186/s13059-015-0622-4)

- 105 Macqueen, D. J., Primmer, C. R., Houston, R. D., Nowak, B. F., Bernatchez, L., Bergseth, S., Davidson, W. S., Gallardo-Escárate, C., Goldammer, T., Guiguen, Y., *et al.* 2017 Functional Annotation of All Salmonid Genomes (FAASG): an international initiative supporting future salmonid research, conservation and aquaculture. *BMC Genomics*. **18**, 484. (doi:10.1186/s12864-017-3862-8)
- 106 Cong, L., Ran, F. A., Cox, D., Lin, S. L., Barretto, R., Habib, N., Hsu, P. D., Wu, X. B., Jiang, W. Y., Marraffini, L. A., *et al.* 2013 Multiplex Genome Engineering Using CRISPR/Cas Systems. *Science*. **339**, 819-823. (doi:10.1126/science.1231143)
- 107 Anzalone, A. V., Randolph, P. B., Davis, J. R., Sousa, A. A., Koblan, L. W., Levy, J. M., Chen, P. J., Wilson, C., Newby, G. A., Raguram, A., *et al.* 2019 Search-and-replace genome editing without double-strand breaks or donor DNA. *Nature*. (doi:10.1038/s41586-019-1711-4)
- 108 Abe, M., Kuroda, R. 2019 The development of CRISPR for a mollusc establishes the formin *Lsdia1* as the long-sought gene for snail dextral/sinistral coiling. **146**, dev175976. (doi:10.1242/dev.175976)
- 109 Yu, H., Li, H., Li, Q., Xu, R., Yue, C., Du, S. 2019 Targeted Gene Disruption in Pacific Oyster Based on CRISPR/Cas9 Ribonucleoprotein Complexes. *Mar Biotechnol (NY)*. **21**, 301-309. (doi:10.1007/s10126-019-09885-y)
- 110 Crawford, K., Diaz Quiroz, J. F., Koenig, K. M., Ahuja, N., Albertin, C. B., Rosenthal, J. J. C. 2020 Highly Efficient Knockout of a Squid Pigmentation Gene. *Current Biology*. **30**, 3484-3490.e3484. (doi:10.1016/j.cub.2020.06.099)
- 111 Perry, K. J., Henry, J. Q. 2015 CRISPR/Cas9-Mediated Genome Modification in the Mollusc, *Crepidula fornicata*. *Genesis*. **53**, 237-244. (doi:10.1002/dvg.22843)
- 112 Potts, R. W. A., Gutierrez, A. P., Cortés-Araya, Y., Houston, R. D., Bean, T. P. 2020 Developments in marine invertebrate primary culture reveal novel cell morphologies in the model bivalve *Crassostrea gigas*. *PeerJ*. **8**, e9180. (doi:10.7717/peerj.9180)
- 113 Tsai, H. J. 2000 Electroporated sperm mediation of a gene transfer system for finfish and shellfish. *Molecular Reproduction and Development*. **56**, 281-284. (doi:10.1002/(sici)1098-2795(200006)56:2+<281::aid-mrd15>3.0.co;2-b)
- 114 Lu, J. K., Chen, T. T., Allen, S. K., Matsubara, T., Burns, J. C. 1996 Production of transgenic dwarf surfclams, *Mulinia lateralis*, with pantropic retroviral vectors. *Proceedings of the National Academy of Sciences of the United States of America*. **93**, 3482-3486. (doi:10.1073/pnas.93.8.3482)
- 115 Cadoret, J. P., Boulo, V., Gendreau, S., Mialhe, E. 1997 Promoters from *Drosophila* heat shock protein and Cytomegalovirus drive transient expression of luciferase introduced by particle bombardment into embryos of the oyster *Crassostrea gigas*. *Journal of Biotechnology*. **56**, 183-189. (doi:10.1016/s0168-1656(97)00118-1)
- 116 Boulo, V., Cadoret, J. P., Shike, H., Shimizu, C., Miyano-hara, A., Burns, J. C. 2000 Infection of cultured embryo cells of the Pacific oyster, *Crassostrea gigas*, by pantropic retroviral vectors. *In Vitro Cellular & Developmental Biology-Animal*. **36**, 395-399.
- 117 Buchanan, J. T., Nickens, A. D., Cooper, R. K., Tiersch, T. R. 2001 Transfection of Eastern Oyster (*Crassostrea virginica*) Embryos. *Marine Biotechnology*. **3**, 322-335. (doi:10.1007/s10126-001-0002-9)
- 118 Kuznetsov, A. V., Pirkova, A. V., Dvoryanchikov, G. A., Panfertsev, E. A., Gavryushkin, A. V., Kuznetsova, I. V., Erokhin, V. E. 2001 Study on the Transfer of Foreign Genes into the Mussel *Mytilus galloprovincialis* Lam. Eggs by Spermatozoa. *Russian Journal of Developmental Biology*. **32**, 254-262. (doi:10.1023/a:1016775303990)
- 119 Guerra, R., Carballada, R., Esponda, P. 2005 Transfection of spermatozoa in bivalve molluscs using naked DNA. *Cell Biology International*. **29**, 159-164. (doi:10.1016/j.cellbi.2004.11.018)
- 120 Chen, H. L., Yang, H. S., Huang, R., Tsai, H. J. 2006 Transfer of a foreign gene to Japanese abalone (*Haliotis diversicolor supertexta*) by direct testis-injection. *Aquaculture*. **253**, 249-258. (doi:10.1016/j.aquaculture.2005.09.017)
- 121 Thresher, R., Grewe, P., Patil, J. G., Whyard, S., Templeton, C. M., Chaimongol, A., Hardy, C. M., Hinds, L. A., Dunham, R. 2009 Development of repressible sterility to prevent the establishment of